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Serological characterization of small ruminant lentiviruses: A complete tool for serotyping lentivirus infection in goat

Chiara Nogarol[a], Luigi Bertolotti[a,*], Siv Klevar[b], Margherita Profiti[a], Britt Gjerset[b], Sergio Rosati[a]

[a] Università di Torino, Dipartimento di Scienze Veterinarie, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy

[b] Norwegian Veterinary Institute, Department of Diagnostics, Section of Immunology, Norway

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ABSTRACT

Small ruminant lentiviruses (SRLVs) form a very heterogeneous group of ssRNA viruses able to infect goats and sheep worldwide. The genetic heterogeneity is reflected by a large antigenic variability which can represents a potential bias in serological diagnostics. Indeed the circulation of four different viral genotypes reveals how the surveillance and control of SRLV is a hard challenge. In previous works we described the use of a single subunit of the viral capsid protein for the SRLV infection genotyping. The amino acid sequence of this region was characterized for each genotype, produced in recombinant form and used as antigen in the described indirect ELISA assay. In this work we completed the panel of antigens including all the divergent genotypes. The subunits of genotypes A, B, C and E were used to test a different groups of goat sera belonging to flocks were the SRLV circulation was proven and genetically characterized. The results confirmed the ability of the P25-B3 subunit to correctly discriminate the viral infection, showing a very high concordance between the SRLV genotype circulating within the flock and the serotype identified by the ELISA test. The proposed approach is able to detect and distinguish all known SRLV genotypes detected so far in Europe. It could represent a cost effective support in SRLV identification, beside the more expensive and time consuming genetic analysis, improving the knowledge about viral heterogeneity. Finally, it may represent a first line epidemiological tool in those Countries in which SRLV have been detected but not yet characterized.

1. Introduction

Small ruminant lentiviruses (SRLVs) form a very heterogeneous group of ssRNA viruses able to infect goats and sheep. SRLV are mainly transmitted from mother to offspring by colostrum and milk ingestion (Pépin et al., 1998), leading to high serum prevalence in absence of specific control programs (Reina et al., 2009a). Genome analyses revealed that SRLV can be divided into at least four divergent genotypes (A, B, C and E) (Grego et al., 2007; Shah et al., 2004b). Genotype A and B include the viral strains historically named Visna Maedi Virus (VMV) and Caprine Arthritis Encephalitis Virus (CAEV) respectively (Shah et al., 2004a). They are distributed worldwide and mainly spread through animal trade. Within A and B genotypes, specific diseases of sheep and goat are associated to particular subtypes (Colitti et al., 2019; Minguijón et al., 2015). Genotypes C and E have been characterized so far in limited geographical areas (Gjerset et al., 2007, 2006; Grego et al., 2007). The former has been identified in Norwegian small ruminant population, while the latter is, to date, strictly associated to some Italian goat populations (Grego et al., 2007; Reina et al., 2010). Several studies were conducted describing antigenic heterogeneity of SRLV variants (de Andrés et al., 2013; Reina et al., 2009c) and emphasizing the impact of such heterogeneity in SRLV diagnostic (Cardinaux et al., 2013; Tavella et al., 2017). Most conventional diagnostic tests are still produced using a single strain-based antigen preparation, especially belonging to MVV like strains, which is believed to detect cross reacting antibodies against epitopes located in major structural proteins (Gogolewski et al., 1985; Rosati et al., 1999). However, a higher sensitivity of antigen preparation, homologous to the infecting strain, has been clearly demonstrated in different studies (Lacerenza et al., 2006; Pépin et al., 1998). Moreover, CAEV- based diagnostic tools were proven to be less efficient at detecting genotype A subtypes in Swiss goats, leading to diagnostic escape, despite of a long eradication campaign (Deubelbeiss et al., 2014). The highly heterogeneous genotype E, identified in Roccaverano breed in north-west Italy (Grego et al., 2007) and Sarda goat in Sardinia (Reina et al., 2010) is another good example. First sequences were obtained by chance in a caprine herd, using a set of degenerated primers designed to amplify a *gag* fragment from the majority of known genotypes, encompassing the

* Corresponding author.
E-mail address: luigi.bertolotti@unito.it (L. Bertolotti).

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major linear capsid. Further analyses revealed the difference between the newly described genotype and the others known, including variation in immunodominant linear epitope within capsid antigen. This aspect highlighted the need of updating the available diagnostic tools (Reina et al., 2009a).

About two decades ago, we characterized a linear epitope of capsid antigen, corresponding to the subunit B3 along the P25 protein sequence, able to discriminate between genotype A and genotype B infections. We proposed the SRLV serotyping as a rapid and cheap tool to identify the circulating strain in a defined population with the aim to drive the use of homologous strain for eradication purposes (Grego et al., 2002; Rosati et al., 1995). Since then, a large number of A and B subtypes and additional genotypes have been identified. The characterization of genotype E (Grego et al., 2007) pushed to update the test panel in order to improve the sensitivity of the available diagnostic tools (Reina et al., 2009c). Even if all the most divergent strains were included in the panel, there is an urgent need to evaluate the diagnostic potential of the proposed serotyping assay, including all known genotypes and subtypes so far identified.

In this study a fourth variant corresponding to Norwegian strain 1GA (acc. Number AF322109) was expressed. The updated set of antigens was used to test a panel of sera from animals infected with all the 4 SRLV genotypes. Results suggest that the identified linear epitope shows an good serotyping potential at the herd level and this test may represent a cost effective tool for a preliminary antigenic characterization of circulating SRLVs in absence of genetic data.

2. Material studied, area descriptions, methods, techniques

A 17 residue polypeptide corresponding to the immunodominant linear epitope of capsid antigen of Genotypes A, B and E have been produced in previous studies (Grego et al., 2002; Reina et al., 2009c; Rosati et al., 1999). In this study a fourth variant corresponding to Norwegian strain 1GA (acc. Number AF322109) was expressed. Amino acidic alignment of the four genotypes has been performed using Clustal W embedded in Geneious software ver. 11.01. Since the genotype C strain was not available, a synthetic construct was obtained by annealing complementary oligonucleotides coding for the 17 residues and carrying at each terminus 4 nucleotides single strand overhang, mimicking the *Bam*HI (GATC) and *Eco*RI (AATT) restriction digestion, to facilitate cloning. All gene fragments were cloned into the pGex-2T expression vector in frame with glutathione S-transferase (GST) (Pharmacia, Uppsala, Sweden). Fusion protein, as well as the GST carrier, were expressed in *Escherichia coli* BL21 and affinity purified (Rosati et al., 1999). The purity and yield of recombinant proteins were estimated by SDS-PAGE and Bradford method (Bradford, 1976).

All recombinant subunits were coated (100 ng/well) on separate wells of Nunc Maxisorp ELISA plates (Thermo Fisher Scientific). To take into account for potential reactivity against carrier moiety, an excess of purified GST was incorporated into sample diluents in order to pre-absorb (if any) anti GST antibodies.

A panel of 293 characterized goat blood sera were included in the present study (Table 1). In order to evaluate the reactivity of each serum against the homologous antigen, a group of sera (n = 163) from animals belonging to Italian flocks infected by SRLV A, B and E and classified in previous studies was used as reference (Grego et al., 2007; Reina et al., 2009c, 2010).

The remaining 130 SRLV positive sera belonging to flocks where genotype C was previously isolated and classified and they were retrieved from frozen collection at the Norwegian Veterinary Institute (Gjerset et al., 2009). Positivity versus SRLV and genotyping assignment of all the Italian samples was proven by genetic analyses based on SRLV *gag* gene amplification and sequencing (Grego et al., 2007), spanning the epitope region. Norwegian sera belonged to flocks where genotype C was isolated and genetically characterized (Gjerset et al., 2009, 2007). The SRLV positivity of all those sera were assessed by commercially available ELISA screening tests (IDEXX CAEV/MVV Total Ab Test).

Table 1
Sample description. N indicates the number of samples tested. The number of positive sera is indicated within brackets. For each sample set the number of correctly typed sera was reported divided by the total number of ELISA positive reactions. The three case of uncorrected typing are starred (*).

Geographic origin	N	SRLV genotype			
		A	B	C	E
Italy	163 (102)	26/26	26/26	0/1*	35/36*
Norway	130 (103)	0/1*	20/20	60/60	–
Total	293 (205)	26/27	46/46	60/61	35/36

Each sample was tested against the four antigen subunits (A, B, C and E) as follows. Samples were diluted 1/20 in sample diluent and incubated (100 µl/well) against each antigen for 1 h at 37 °C. Following three washes, 10 ng of peroxidase-labeled anti sheep/goat IgG was added (100 µl/well) and the plate was incubated as described above. After final washing step, the reaction mixture was developed with 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid) (Chemicon, Temecula, Calif.). ELISA absorbances were obtained for each serum sample against the four subunits. The results were read at 405 nm and absorbances were compared, among antigens, for each sample.

Conservative cutoffs were chosen, in order to clearly identify a specific reactivity and avoiding possible misinterpretations: i) a minimum absorbance of 0.4 was used to allow the serotyping and ii) a difference of at least 140% in reactivity between antigens was needed to identify the infecting SRLV. In more details, the proposed results interpretation is based on the analysis of the reactivity showed by sera against all the antigens. The main hypothesis is that animals can react in a stronger manner against homologous antigens (i.e. the antigens presented by the infecting viral strain), as previously demonstrated (Reina et al., 2009c). The comparison between the two most reactive antigens allowed the identification of the infecting SRLV viral strain. On the other hand, if the difference between the two most reactive antigens was too low the sample serotype was not determined (reported in gray in Fig. 1). The concordance between the SRLV genotype circulating within the flock and the serotype identified by the ELISA test was assessed by Cohen's Kappa evaluation in a 4 × 4 matrix with R statistical software (R Core Team, 2015).

3. Results

Analysis of the capsid antigen subunits alignment of the four SRLV genotypes revealed how A, B and C peptides showed a high similarity in the first half of the considered region whereas the second half of the region of the genotype C subunit was more similar to the genotype E (Table 2). Variation within genotype at the protein level was much less evident, suggesting that the proposed four recombinant polypeptides may be useful markers for all known subtypes (Table 2). Italian sera results are showed in Fig. 1. In all cases, sera reacted in a specific manner against the homologous antigen. Sera from the Norwegian flock were tested against the four antigens and a clear reactivity against B and C antigens was evident (Fig. 2).

Among all the 293 sample, 205 were considered as positive showing a reactivity greater than 0.4 against at least one out the four antigens (69.97%).

One hundred and seventy out of the 205 positive sera could be assigned to a defined genotype (82.93%). The results are summarized in Table 1. The remaining 35 sera showed a small difference between the two highest reactivities to allow a good discrimination. The 98.23% of

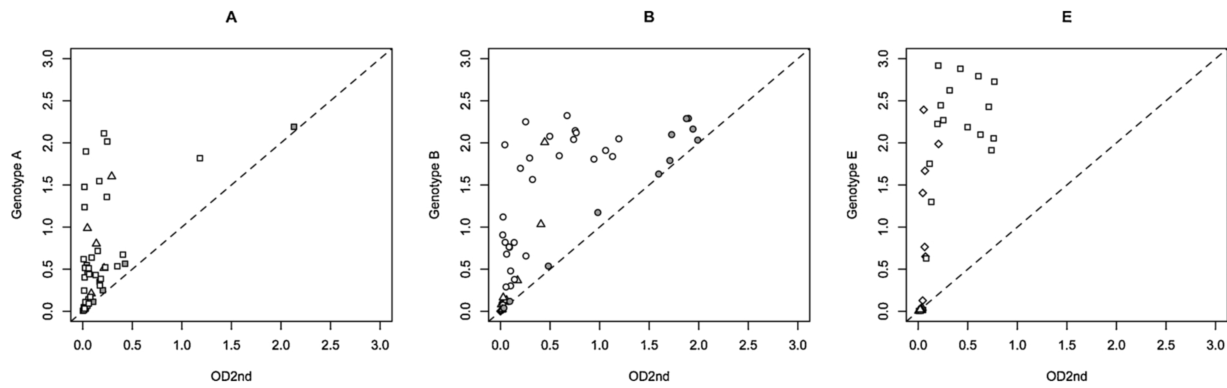

Fig. 1. Italian sera. The sera belonging to genotypes A, B and E positive flocks were tested against the homologous and heterologous antigens. The y axis reports the reactivity of the sera against the antigen A, B and E. The reactivity of the second most reactive antigen is reported on the x axis. Sera with not determinable reactivity are in gray. The dashed line represents the perfect cross-reactivity.

the genotyped sera were correctly classified, according to *gag* sequence, showing the highest reactivity versus the homologous antigen. Only three sera were misclassified: 1 sample from a genotype A positive Italian flock was classified as E, 1 sample from a genotype E positive Italian flock was classified as C and finally 1 sample from a C positive Norwegian flock was classified as A. In the first two incorrect classifications the maximum absorbances were slightly above the positivity cutoff (0.448 and 0.452 respectively) whereas in the latter case, the ratio between OD1st and OD2nd was very close to the proposed discrimination limit (OD^A = 2.601, OD^C = 1.732, ratio = 1.501732). The final concordance between SRLV genotype and ELISA results was equal to 0.976 (95% CI: 0.949–1.000).

4. Discussion

This study demonstrates that a linear epitope of the capsid antigen of SRLV is capable to differentiate all four known genotypes according to sequence variation and specific antibody reactivity. Based on *in silico* evaluation, we provided an updated analysis of amino acid sequence of all described subtypes spanning the epitope region (Table 2). This describes a clear epitope variation among genotypes and a certain degree of epitope conservation within each genotype. According to previous study (Rosati et al., 1999), 17 residues likely encompass two consecutive linear epitopes, being the most N'- terminal immunodominant. According to Table 2, genotypes A, B and C share the same C' terminal sequence (KLNEEAERW, while genotype E shows two substitution in position 4 and 8. The N'-terminal part of polypeptide, as expected, is variable among genotypes but, surprisingly, is highly similar between genotype C and E, sharing the sequence RQNPQP. According to serology, a certain degree of cross-reactivity was only found between genotype C and genotype E infected goats further suggesting that the N' terminal part of the peptide could be relevant for discrimination.

Sensitivity of the serological assay described here was lower than those obtained in previous works (Reina et al., 2009b; Rosati et al.,

Table 2
Sequence of the P25-B3 subunit of all available SRLV genotypes and subtypes. Genbank accession numbers are reported within brackets. Dots indicates identity, using A1 sequence as reference. Most similar regions between genotype C and B, and between genotype C and E are boxed.

Subtype	Sequence	Alignment
A1 [M60609]	KLNEEAERWVRQNPPGP	KLNEEAERWVRQNPPGP
A2 [AY101611]	KLNEEAERWVRQNPPGP	....
A3 [KY358787]	KLNEEAERWVRQNPPGP	....
A4 [AY445885]	KLNEEAERWVRQNPPGP	..............A..
A4[KT453989]	KLNEEAERWLRQNPPGP	.........L.......
A5 [AY454175]	KLNEEAERWVRQNPPGP	....
A8 [EF676006]	RLNEEAERWVRQNPPGQ	R...............Q
A9 [EF676018]	KLNEEAERWVRQNPPGP	....
A10 [AY265455]	KLNDEAERWVRQNPPGP	...D.............
A11 [FR693815]	KLNEEAERWVRQNPPGP	....
A12 [FJ623123]	KLNEEAERWVRQNPPGP	....
A13 [FJ623120]	KLNEEAERWVRQNPPGP	....
A17 [MK348421]	KLNEEAERWVRQNPPGP	....
A18 [MH374287]	KLNEEAERWVRQNPPGP	....
A19 [MG5544009]	KLNEEAERWIRQNPPGP	.........I.......
B1 [M36677]	KLNEEAERWRRNNPPPP	KLNEEAERWRRNNPPPP
B2 [FJ95346]	KLNEEAERWRRNNPPPP	.........R.N...P.
B2 [AY265456]	KLNEEAERWRRNNPPPQ	.........R.N...PQ
B3 [JF502416]	KLNEEAERWRRNNPPPA	.........R.N...PA
C [AF322109]	KLNEEAERWRRQNPQPA	KLNEEAERWRRQNPQPA
E1 [EU293537]	KLNKEAETWMRQNPQPP	KLNKEAETWMRQNPQPP
E2 [GQ381130]	KLNKEAETWMRQNPQPP	...K...T.M....QP.

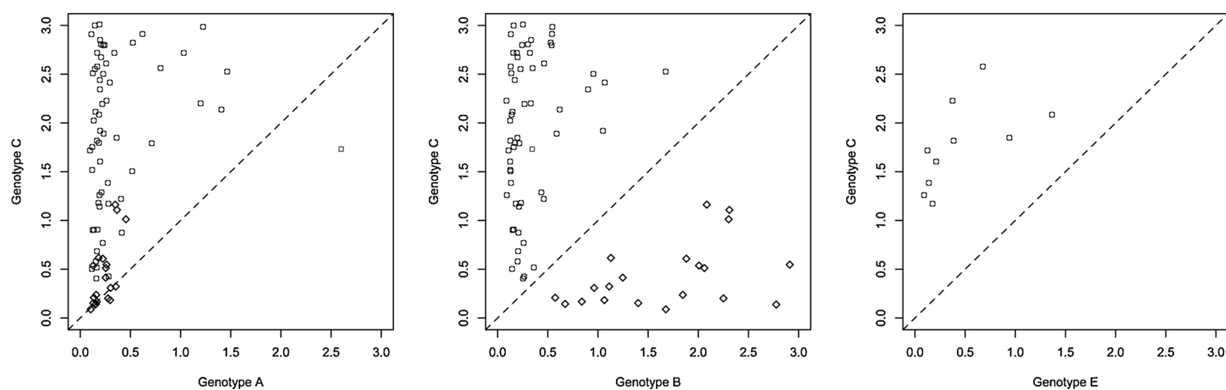

Fig. 2. Norwegian sera. Each serum was tested against the four SRLV epitopes. The reactivity of each serum against the genotype C epitope is showed on the y axis; the reactivities against the other 3 genotypes are reported on the x axis of the three panels. The shape of each points indicates the most reactive antigen: A (square), B (diamond), C (circle), E (triangle). The dashed line represents the perfect cross-reactivity.

1999). This can be easily explained if we consider that serological screening tests usually are based on multiepitope antigens, whereas the proposed genotyping test is based on a single epitope. Moreover the proposed test should not be intended as confirmatory test at individual level. Previous investigation suggested that the genotyping test seems to be enough informative if 5–10 screening positive samples/flock are tested and serotyped. This step could be suggested as a potential strategy where no information about SRLV circulating strain are available.

About the 83% of the samples showing reactivity against at least one antigen were serotyped and the concordance between serotyping and genotyping was greater than 97%. Concerning Norwegian samples a detailed genetic characterization of serum panel was not available. However all the samples belonged to herds were the genotypes B and C where previously characterized. The proposed serological test was able to detect and correctly classify positive samples in this serum panel. Information on genetic and/or antigenic properties of SRLV in many European Countries, especially on the North side, is still missing. However, due to increasing interest to dairy goat industry, there is a need to fill the gap in order to implement adequate diagnostic tools. In fact, the need for serotyping SRLV infection is important especially in small ruminant population where genetic information is lacking. Moreover, serotyping the positive animals was recently identified as a potential strategy for the identification of virulent B1 strains (De Martin et al., 2019), that can help in the eradication process of SRLV in Switzerland.

5. Conclusion

Due to extensive antigenic heterogeneity among genotypes, the use of appropriate diagnostic assays could represent a key of success in future control programs. In this context the availability of a serotyping method able to detect and distinguish all known serogroups detected so far in Europe could represent a cost effective method, alternative to more expensive and time consuming genetic analysis. Finally, it may represent a first line epidemiological tool in those Countries in which SRLV is detected but not yet characterized.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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